RNAi technology and its use in studying the function of nuclear receptors and coregulators

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Until just a few years ago, RNA interference (RNAi) technology was restricted to the research fields of plants, C. elegans or Drosophila. The discovery of gene silencing by in vitro synthesized double-stranded RNA (dsRNA) in mammalian cells has made the use of RNAi possible in nearly the entire life science kingdom. DNA vectors delivering small interfering RNA (siRNA) directed by polymerase III or polymerase II promoters to persistently inhibit target genes expression have extended this technology to study in vivo function of these genes. Recently, RNAi has been used as a powerful tool in the functional analysis of nuclear receptors and their coregulators. This short review will cover studies in this area.

Received July 16th, 2003; Accepted August 26th, 2003; Published September 10th, 2003 | **Abbreviations:** **RNAi:** RNA interference; **shRNA:** Short hairpin RNA; **siRNA:** Small interfering RNA |

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Introduction

RNA interference (RNAi) was first discovered in the nematode worm *Caenorhabditis elegans* as a biological response to double-stranded RNA (dsRNA), which resulted in potent sequence-specific gene silencing [Fire et al., 1998]. RNAi is an evolutionarily conserved process involving a multi-step event, which generates small interfering RNAs (siRNAs) of 21- to 23-nucleotide (nt) *in vivo* by endogenous RNase III enzyme-Dicer. The resulting siRNAs mediate destruction of their complementary mRNA. The biology and mechanisms of RNAi have been reviewed recently in detail [Fire et al., 1998; Hannon, 2002]. The breakthrough of RNAi study is the discovery that dsRNA can selectively suppress gene expression in cultured mammalian cells through RNAi [Caplen et al., 2001; Elbashir et al., 2001a]. The short (<30 nt) synthetic interfering RNA duplexes are successfully used to induce sequence-specific gene silencing yet evade the host interferon response which usually is activated by dsRNA longer than 30nt [Baglioni and Nilsen, 1983; Williams, 1997]. Based on the biochemical analysis of siRNA in *Drosophila* [Elbashir et al., 2001b; Elbashir et al., 2001c; Zamore et al., 2000], the structure of the *in vitro* synthesized siRNA is found to be important to achieve effective gene inhibition [Elbashir et al., 2001a]. SiRNAs with 3′ overhangs of two uridines have been found to be more efficient in cultured mammalian cells [Elbashir et al., 2001c]. At the moment, there is no criterion to predict the ideal target sequence of an siRNA. Although many of the siRNAs reported to date are designed to target coding sequences, especially the amino terminus 100-200 bases away from AUG [Sui et al., 2002], successful gene silencing has been reported for siRNA by targeting various sequences, including the 3′ untranslated region [McManus et al., 2002]. Therefore, the target sequences published in the literatures will definitely provide a candidate pool for scientists who are interested in the RNAi research.

A major drawback of using *in vitro* synthesized siRNAs is its transient nature because mammalian cells lack the mechanism to amplify siRNA-mediated silencing observed in *C. elegans* and *Drosophila* [Hannon, 2002]. Gene expression was only suppressed for no more than one week. In the beginning of 2002, DNA vectors expressing siRNAs directed by RNA polymerase III promoters were made by several groups [Brummelkamp et al., 2002; Paddison et al., 2002; Sui et al., 2002]. These expression vectors mediate the production of siRNAs from transcripts containing a stem and loop structure-short hairpin RNA (shRNA), which will lead to the continued expression of siRNAs in the cells with a persistent and specific knock-down of the target genes. This improved expression system paves the way for long-term loss-of-function studies.

However, the Polymerase III promoter is active in all tissues and cannot be used to generate the tissue-specific knock-down. Just recently, a new vector expressing long ds-RNA from the Polymerase II promoter has been developed to knock-down the target gene [Shinagawa and Ishii, 2003]. The ds-RNA transcribed from this vector lacking 5′-cap structure and 3′-poly(A) tail, which facilitate its exportation to cytoplasm, can silence target gene without producing the interferon response. Transgenic mice embryos expressing long ds-RNA for the transcriptional co-repressor Ski from this vector exhibited similar phenotypes to those of Ski-knock-out embryos. Therefore, with this polymerase II expression vector, one can efficiently knock down the expression of any gene in animal in a tissue-specific manner without the host interferon response.

RNAi for nuclear receptors and coregulators

RNA interference with synthesized dsRNA or vector-based siRNAs has endowed research with a whole set of tools that facilitate genetic studies of many fields, including the recent application of RNAi approaches in

Method	Receptor/ Co-regulator	Target Sequences	Effectiveness % inhibition	Reference
Synthetic	FXR	5'-GGG GAU GAG CUG UGU GUU G-3'	50	Plass et al, 2002
dsRNA	AR	5'-AAG CCC AUC GUA GAG GCC CCA-3'	>95	Wright et al, 2003
	SRC-1	5'-AAC ACG ACG AAA UAG CCA UAC-3'	80-90	Li et al, 2003
		5'-AAG UGA UGA CUC GUG GCA CUG-3'	80-90	Li et al, 2003
		5'-CCU CAG GGC AGA GAA CCA UCU-3'	80-90	Shang and Brown, 2002;
	SRC-2	5'-AAG UCA GAU GUA UCC UCU ACA-3'	80-90	Li et al, 2003
	SRC-3	5'-AGA CUC CUU AGG ACC GCU U-3'	>90	Li et al, 2003; Shang and Brown, 2002; Zhou et al, 2003
		5'-GAC UCC UUA GGA CCG CUU U-3'	>90	**
		5'-UCG AGA CGG AAA CAU UGU A-3'	>90	**
	p300	5'-AAC CCC UCC UCU UCA GCA CCA-3'	80-90	Debes et al, 2002
	NCoR	5'-AAU GCU ACU UCU CGA GGA AAC A-3'	50-60	Yoon et al, 2003
	SMRT	5'-AAG GGU AUC AUC ACC GCU GUG-3'	>95	Yoon et al, 2003
		5'-AAU GAG CCU GAA UAC AAU AUC-3'	>95	*
	HDAC3	5'-AAU AUC CCU CUA CUC GUG CUG A-3'	>95	Yoon et al, 2003
		5'-AAU CAG AAC UCA CGC CAG UAU-3'	>95	*

Table 1. siRNA targeting sequences of nuclear receptors and co-regulators Abbreviations: FXR, Farnesoid X receptor; AR, Androgen Receptor; SRC-1/2/3, Steroid Receptor Coactivator-1/2/3; NCoR, Nuclear Receptor Co-Repressor; SMRT, Silencing Mediator for Retinoid and Thyroid hormone receptor; HDAC3, Histone Deacetylase 3; TBL1, Transducin β-Like protein 1; TBLR1, Transducin β-Like protein 1-Related protein; ERRα, Estrogen Related Receptor α; ARA55, AR-associated protein 55; ARA70, AR-associated protein 70. * Ho-Geun Yoon and Jiemin Wong, personal communication. ** Haijun Zhou unpublished results

functional analysis of nuclear receptors and their co-regulators. Since last year, RNAi has been introduced to silence the expression of nuclear receptors and their co-regulators in transient transfection assays (see Table 1 for details). FXR [Plass et al., 2002] and AR [Wright et al., 2003] are the only published examples of nuclear receptor superfamily which have been successfully inhibited with synthetic siRNA. For the nuclear receptor co-regulators, RNAi has been more widely used. By introduction of synthetic siRNA, co-activators SRCs [Li et al., 2003; Shang and Brown, 2002; Zhou et al., 2003] and p300 [Debes et al., 2002] and co-repressors NcoR and SMRT [Yoon et al., 2003] have been shown to be inhibited efficiently. DNA vector based RNAi has been demonstrated by several groups to knock down ERR α [Schreiber et al., 2003] and the AR coactivators ARA55 [Rahman et al., 2003a] and ARA70 [Rahman et al., 2003b]. Usually, the change of target gene expression is detected by western analysis. In the case of FXR and ERR α , RNA levels were determined [Plass et al., 2002; Schreiber et al., 2003], while immunocytochemistry was used to detect p300 after silencing by siRNA [Debes et al., 2002].

Comparison of RNAi with other techniques

Distinct from the antisense oligodeoxynucleotides (asODN) and dominant negative forms, which mediate RNaseH cleavage and functional interference respectively, RNAi leads to target gene mRNA degradation through the RNAase III machinery resulting in the knock-down of protein expression, thereby affecting the functions of nuclear receptors and coregulators. Although RNAi technology has not been applied in any *in vivo* system of nuclear receptors or coregulators, RNAi induced effects in *in vitro* studies are comparable or better than those produced by antisense oligodeoxynucleotides [Cavarretta et al., 2002] or dominant negative form [Rahman et al., 2003a; Rahman et al., 2003b]. In these studies, asODN to SRC-1 reduced SRC-1 protein level by 78% at best. In contrast, RNAi to SRC-1 is able to reduce 80%-90% of its protein level. In addition, ARA55 and ARA70 knocking-down with DNA vector-based RNAi, reduced the expression of AR target gene, PSA, to a similar extent as that reduced by ARA55 and ARA70 dominant negative mutants. For the reporter assay, RNAi knocking-down of ARA55 and ARA70 exerted a greater

inhibition of MMTV promoter activity than those produced by dominant negative forms of ARA55 and ARA70. In general, the effectiveness in most cases (listed in Table 1) is dramatic and has greatly helped the functional analysis of these proteins.

Conclusion

There is no golden principle to predict the length of sustaining time of RNAi inhibition, because each protein has its own turnover time. Normally, the transient effect of siRNA inhibition lasts less than one week. Multiple transfections may be needed to extend its effect to a longer time. Otherwise, vector-based RNAi either by polymerase II or III is required to archive permanent knock-down of target gene expression. The length of targeting sequences range from 19nt to 21 nt, which guarantee the sequence specificity, without generating interferon response and mimic the product length of Dicer. Usually, the effective sequence is chosen from several candidates; therefore, the targeting sequences listed here will be very helpful for the functional analysis of nuclear receptors and co-regulators. Although RNAi technology has not been used as widely as it can be in the nuclear receptor field, we believe the importance and effectiveness of this technology will be increasingly used for functional analysis of nuclear receptor and coregulator function for the years to come.

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